Plant Physiol. (1988) 86, 626-630
0032-0889/88/86/0626/05/$01.00/0

Hydrogen Peroxide Metabolism in Soybean Embryonic Axes at the Onset of Germination[1]

Received for publication January 15, 1987 and in revised form April 30, 1987

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ABSTRACT

Hydrogen peroxide steady state levels of 5 micromolar were determined in soybean (*Glycine max*) embryonic axes incubated for 2 hours and in axes pretreated with aminotriazole or cyanide, where these levels were 50 and 1 micromolar, respectively. The activities of catalase (105 picomoles H_2O_2 per minute per axis), peroxidase (10–44 picomoles H_2O_2 per minute per axis), glutathione peroxidase (3 picomoles H_2O_2 per minute per axis) and superoxide dismutase (3.5 units per axis), were also determined. Catalase seems to be the most important H_2O_2 consuming enzyme at the physiological concentration of H_2O_2. A short treatment with aminotriazole, while substantially increasing H_2O_2 level, did not affect the growth of the axes. The production of superoxide anion by the mitochondria isolated from soybean axes was measured from the superoxide dismutase-sensitive rate of adrenochrome formation in the presence of NADH or succinate as substrate and amounted to 1.3 and 0.8 nanomole O_2^- per minute per milligram protein, respectively. According to the stoichiometry of O_2^- and H_2O_2 dismutation reactions, it is apparent that about 0.9 to 1.5% of the total oxygen uptake proceeds through the formation of the free intermediates of the partial reduction of oxygen.

The physiological and biochemical events taking place during the initial stages of seed imbibition have been the subject of numerous studies (3, 16). A number of processes such as membrane reorganization (29) and metabolic reactivation (2, 20, 26) occurring at this stage can have profound influence on seed germination and on the future growth of the seedling (21, 34).

Hydroperoxide metabolism in plants in general and particularly during seed germination has received little attention, although information about hydroperoxide production and utilization could be most relevant to understand important processes such as lipid peroxidation and NADPH redox changes. Several authors have indicated that an increased hydroperoxide production could be linked to a shift to the oxidized state of the NADPH/$NADP^+$ couple (7, 15, 25, 31). Taylorson and Hendricks (32), in order to explain the role of cyanide in breaking seed dormancy, proposed that the inhibition of catalase by cyanide could result in a higher steady state level of H_2O_2 which would lead, through an enhanced peroxidase activity, to a lower NADPH/$NADP^+$ ratio and to a stimulation of the pentose phosphate pathway. Such an interpretation has three implicit assumptions: (a) that there is a significant H_2O_2 production early in the germination process; (b) that catalase utilizes an important fraction of the H_2O_2 produced; and (c) that both H_2O_2 production and H_2O_2 utilization to oxidize NADPH are cyanide insensitive.

In this paper we report a measurement of H_2O_2 production and of the activities of the enzymes involved in H_2O_2 metabolism at an early stage of soybean embryonic axis imbibition.

MATERIALS AND METHODS

Soybean seeds (*Glycine max*, cv Hood) recently harvested, were air-dried to 12% humidity at room temperature and stored at 20°C in an evacuated desiccator until used; germination capacity remained over 92% throughout.

Measurements of H_2O_2. Embryonic axes incubated for 2 h at 26°C over water-saturated filter paper were used for the determination of H_2O_2 steady state levels. The diffusates were prepared by soaking 80 axes in 3 ml of 40 mM potassium phosphate buffer (pH 7.0). Two aliquots of 0.3 ml were taken at the indicated times; one of them was supplemented with 10 μM catalase for 10 min, and H_2O_2 was assayed in both aliquots by measuring the peak values of chemiluminescence produced in the reaction of H_2O_2 with luminol (28, 33). The reaction was carried out in a scintillation vial containing 2.70 ml of 40 mM potassium phosphate buffer (pH 7.8), 20 μM $FeSO_4$, and 10 μM luminol (Sigma). Light emission was measured, for 30 s, immediately after adding the aliquots to the luminol solution and analyzed in a Packard Tri-Carb model 3320 scintillation counter in the out-of-coincidence mode. Reagents and vials were dark-adapted before use.

When the embryonic axes were previously incubated with aminotriazole, aliquots of 0.03 ml were taken, diluted to 0.3 ml and processed as described before. When the axes were incubated in 16 or 160 μM H_2O_2 solutions, samples of 0.1 ml or 0.01 ml were diluted to 0.3 ml and processed as described.

Alternatively, H_2O_2 concentration in the diffusates was measured by formation of the enzyme-substrate complex of horseradish peroxidase (5).

Isolation of Mitochondria. Mitochondria were isolated from (1000–2000) soybean embryonic axes that were incubated on filter paper covering wet cotton for 2 h at 26°C following the procedure described by Bonner (4) and modified according to Beconi *et al.* (1). The axes were homogenized in 150 ml of 0.3 M mannitol, 1 mM EDTA, 4 mM cysteine, and 5 mM Tris-HCl (pH 7.0), in a mortar during 4 min and passed twice through a Potter-Elvehjem homogenizer. The homogenate was filtered through three layers of cheesecloth and centrifuged at 18,000*g* for 10 min in a Sorvall RC-2 centrifuge to sediment mitochondria. The sediment was suspended in 20 ml of 0.45 M mannitol, 1 mM EDTA, and 5 mM Tris-HCl (pH 7.0), homogenized and centrifuged at 270*g* for 10 min to sediment nuclei, cell debris,

[1] Supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina (CONICET). R. A. S. and A. B. are Career Investigators from CONICET.

cellulose fibers, and so forth. The supernatant was centrifuged at 14,500*g* for 10 min. The mitochondrial pellet was washed twice and the final suspension (8–12 mg of protein/ml) was made with the same mannitol-EDTA-Tris solution. All operations were performed at 0–2°C.

Enzyme Assays. For the measurement of enzyme activities, dissected embryonic axes were incubated for 2 h on wet filter paper at 26°C and then homogenized in 50 mM potassium phosphate buffer (pH 7.0), followed by centrifugation at 700*g* for 10 min, at 0 to 2°C. The supernatant was used as the source of enzymes. All enzymes were assayed at 26°C.

Superoxide dismutase activity was assayed spectrophotometrically at 480 nm by the method of Misra and Fridovich (17), in a reaction medium containing glycine-NaOH buffer 50 mM (pH 9.0) and 1 mM epinephrine. The amount of homogenate inhibiting by 50% the oxidation of epinephrine to adrenochrome was taken as 1 unit (adrenochrome assay) of superoxide dismutase activity. Under the conditions of the assay, this amounts to 0.12 μg of superoxide dismutase/ml.

Ascorbate peroxidase and dehydroascorbate reductase activities were assayed spectrophotometrically (E_{265} ascorbate = 15 $mM^{-1} \cdot cm^{-1}$). The reaction medium for ascorbate peroxidase consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.05 mM ascorbate, 0.1 mM H_2O_2, and 0.1 mM EDTA. A correction was made for the nonenzymic rate of ascorbate oxidation by H_2O_2 (19, 23). The reaction medium for dehydroascorbate reductase consisted of 50 mM potassium phosphate buffer (pH 7.0), 1.5 mM GSH, 0.2 mM dehydroascorbic acid, and 0.1 mM EDTA. Reaction rates were corrected for nonenzymic reduction of dehydroascorbate by GSH (18, 19).

Peroxidase activity was measured spectrophotometrically at 430 nm (E = 2.47 mM^{-1} cm^{-1}) in a reaction medium consisting of 50 mM potassium phosphate buffer (pH 7.0), 5 mM pyrogallol, and 1 mM H_2O_2 (27).

Glutathione peroxidase activity was measured spectrophotometrically at 340 nm. The assay mixture contained 0.5 mM *t*-butyl-hydroperoxide, 1 mM GSH, 0.1 unit/ml glutathione reductase, and 200 μM NADPH in 50 mM potassium phosphate buffer (pH 7.0) (12).

Catalase was assayed by back titration with 0.05 N $KMnO_4$ of the remaining H_2O_2 in a reaction medium consisting of 50 mM phosphate buffer (pH 7.0), 60 mM sodium perborate. Aliquots of the assay mixture were taken after 30, 60, and 90 s and the reaction was stopped by addition of 1 M H_2SO_4 (11).

Other Determinations. Determination of O_2 uptake of isolated soybean embryonic axes were made polarographically using a Clark oxygen electrode, in a reaction medium consisting of 50 mM potassium phosphate buffer (pH 7.0).

The effect of H_2O_2 and aminotriazole on the growth of the seedlings was performed by incubating the axes with H_2O_2 and aminotriazole for 15 min; afterwards the axes were removed and placed on wet paper and weighed after 48 h.

Production of superoxide radical was determined as the superoxide dismutase-sensitive rate of adrenochrome formation, measured at 485 to 575 nm (E = 2.97 mM^{-1} cm^{-1}) in a Perkin-Elmer dual wavelength spectrophotometer. The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, 30 mM Tris-HCl (pH 7.0), and 1 mM epinephrine (5).

RESULTS

The light emission of the reaction of H_2O_2 and luminol allowed us to measure the amount of H_2O_2 diffused out of soybean embryonic axes. The kinetics of the reaction showed maximal luminescence in the first 30 s after the reactants were mixed (Fig. 1); therefore all determinations were made at this initial time interval and considering emission peak values. Preincubation of both H_2O_2 standard solutions and aliquots of the diffusate from the axes with catalase removed most of the signal showing that H_2O_2 was the main reactant for light emission and that other molecular species capable of reacting with luminol were quantitatively less important (Fig. 1).

The catalase-sensitive luminol emission of diffusate aliquots

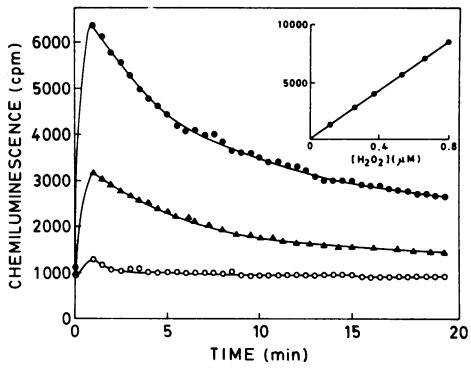

FIG. 1. Luminol-dependent chemiluminescence in H_2O_2 solutions and diffusate from soybean embryonic axes: 0.5 μM H_2O_2 (●); 2 min-incubated diffusate (▲); 2 min-incubated diffusate treated with 10 μM catalase (○). Inset: Luminol-dependent and catalase-sensitive chemiluminescence of H_2O_2 solutions. The abscissa values indicate H_2O_2 concentrations in the reaction mixture in the scintillation vials.

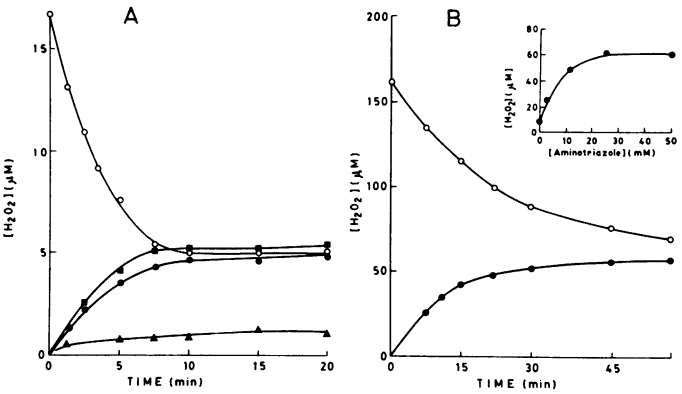

FIG. 2. H_2O_2 concentrations in diffusates from soybean embryonic axes and external incubation medium. A, H_2O_2 in the diffusate (●); H_2O_2 in the diffusate in the presence of 0.1 mM cyanide (▲); and H_2O_2 in the external medium when the soybean embryonic axes were initially added with 16 μM H_2O_2 (○). H_2O_2 in the diffusate measured by formation of horseradish peroxidase-H_2O_2 compound (■). B, H_2O_2 in the diffusate (●) and external incubation medium (○) when the soybean embryonic axes were pretreated with aminotriazole. Inset: H_2O_2 equilibrium concentration in the external medium as function of aminotriazole concentration.

Table I. *Effect of Aminotriazole and H_2O_2 in the Growth of Soybean Seedlings*

Treatment	Concentration	Seedling Weight
	mM	*mg*
Control		35.3 ± 0.7
Aminotriazole	0.1	36.1 ± 0.8
	10	35.7 ± 0.7
	50	36.7 ± 0.6
	100	33.3 ± 0.9
H_2O_2	0.8	32.5 ± 0.5
	4.1	32.2 ± 0.4
	8.3	34.5 ± 0.8
	8300	13.2 ± 0.8

increased with the time of incubation up to a maximum equivalent to 5 μM H_2O_2 in the diffusate; this situation was reached in about 8 to 10 min. In this condition the diffusate apparently reached an equilibrium with the intracellular steady state level of H_2O_2 (Fig. 2A). Similar results were obtained when the H_2O_2 in the diffusate was measured by formation of the enzyme-substrate complex of horseradish peroxidase; again the steady state concentration of H_2O_2 was about 5 μM. When the axes were incubated with 16 μM H_2O_2 in the external medium, the H_2O_2 concentration measured by the luminol assay decreased with time to reach a concentration of about 5 μM with a t one-half of 4 min (Fig. 2A). These results indicate that in this case H_2O_2 in the external medium reaches also a diffusion equilibrium with the cell internal medium and that the steady state concentration of H_2O_2 in the soybean axes is about 5 μM.

Cyanide, which inhibits catalase activity as well as the mitochondrial production of H_2O_2 (see below), severely depressed the H_2O_2 steady state concentration (Fig. 2A).

The pretreatment of the axes during 15 min, with aminotriazole (an irreversible inhibitor of catalase [22]) before assaying for H_2O_2, significantly increased the H_2O_2 in the diffusate (Fig. 2B). The H_2O_2 in the diffusate of aminotriazole-treated axes reached a level of about 50 μM which seems to be the steady state concentration of H_2O_2 in the axes when this inhibitor supressed catalase activity. This effect of aminotriazole (Fig. 2B, inset) shows the importance of catalase in controlling the steady state level H_2O_2. Aminotriazole-treated axes incubated in an external medium containing 0.16 mM H_2O_2 decreased this level to 50 μM H_2O_2 with a t one-half of 21 min (Fig. 2B).

The treatment with aminotriazole did not affect the growth of the axes (Table I). Treatment with up to 100 mM H_2O_2 did not affect axis growth, which may be explained by the rapid exodiffusion of H_2O_2 and by H_2O_2 utilization.

Figure 3A shows O_2^- production by the isolated mitochondria in the presence of NADH which functions as substrate of the NADH dehydrogenase of the external side of the inner mitochondrial membrane (10). The rate of O_2^- production was calculated from the superoxide dismutase-sensitive rate of adrenochrome formation.

The traces of Figure 3A also show that cyanide inhibits O_2^- production by mitochondria in agreement with similar observations in animal and plant mitochondria (13, 24). Figure 3B shows the rate of O_2^- production in the presence of succinate and glutamate as substrates.

The activities of catalase and other H_2O_2 consuming enzymes were measured in homogenates of soybean embryonic axes (Table II). Figure 4 gives an integrated picture of the various metabolic pathways related to the production and utilization of H_2O_2

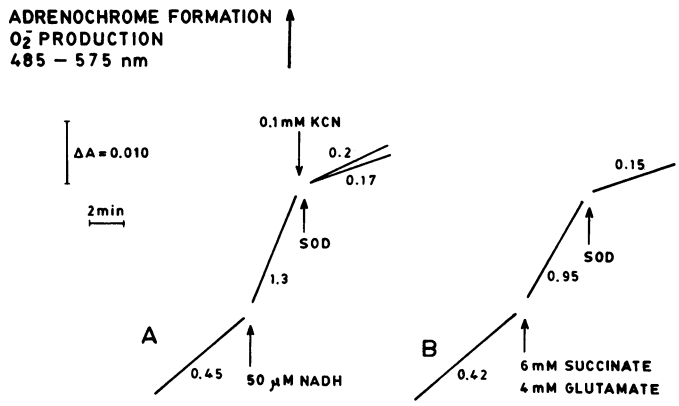

FIG. 3. Production of O_2^- by mitochondria isolated from soybean embryonic axes; 0.59 mg protein/ml. The numbers near the traces indicate the rate of O_2^- production in nmol/min·mg protein.

Table II. *Activities of Enzymes Involved in H_2O_2 Metabolism*

Enzyme	Activity
	pmol H_2O_2/min·axis
Catalase[a]	105
Peroxidase (pyrogallol)	44
Peroxidase (ascorbate)	10
Glutathione peroxidase	3
Superoxide dismutase[b]	115
Dehydroascorbate reductase	12
Oxygen uptake (pmol O_2/min·axis)	4200

[a] Assuming H_2O_2 in the axes equal to 5 μM. [b] Assuming O_2^- in the axes equal to 0.1 nM. Corresponds to 3.5 units/axis (17).

according to the results presented in this paper. The rate of mitochondrial O_2^- production (230 pmol/min·axis) was calculated from the specific rate of O_2^- production in the presence of external NADH as substrate (Fig. 3A) and the amount of mitochondrial membranes in the axis (0.19 mg protein/axis). This rate of O_2^- production will provide a rate of 115 pmol H_2O_2/min·axis according to the stoichiometry of the dismutation reaction. Peroxidase activity using either pyrogallol or ascorbate as hydrogen donor could utilize either 44 or 10 pmol H_2O_2/min·axis, respectively. Glutathione peroxidase could be able to use 3 pmol H_2O_2/min·axis. All these rates were calculated assuming maximal levels of hydrogen donor.

In the case that mitochondrial H_2O_2 was the only significant H_2O_2 source and that glutathione peroxidase and peroxidase (ascorbate as donor), acted at maximal H_2O_2-utilizing rates, catalase would use the rest of H_2O_2 production, *i.e.* 102 pmol H_2O_2/min·axis. If utilization of H_2O_2 by catalase occurs according to d-$[H_2O_2]/dt = k$-$[H_2O_2]$ [Cat] (7) with $k = 4.6 \times 10^7$ M^{-1} s^{-1} and the steady state value of $[H_2O_2]$ equal to 5 μM, the rate of H_2O_2 utilization amounts to 105 pmol H_2O_2/min·axis.

DISCUSSION

The results of this study indicate that H_2O_2-producing and H_2O_2-consuming processes are active early in the germination of soybean embryonic axes. Mitochondrial electron transfer appears to be the most important H_2O_2 source. Mitochondrial H_2O_2 is generated through the primary production of O_2^- and its subsequent dismutation (13). The rate of O_2^- production measured in mitochondria isolated from soybean axes agrees with values found in submitochondrial particles from potato tubers (6), mung bean hypocotyls (24), and *Arum maculatum* spadices (14). These values also agree with the rates of H_2O_2 production measured in mitochondria isolated from potato tubers and mung bean hypocotyls (13, 23).

From the ratio of O_2^- production and total O_2 uptake (Table II) it can be estimated that about 3.6 to 5.9% of the electron flow in mitochondria (extramitochondrial O_2 uptake is not considered) results in univalent reduction of oxygen, depending on whether succinate-glutamate or external NADH are considered the physiological mitochondrial substrate. Due to the stoichiometry of O_2^- and H_2O_2 dismutations (6) it follows that about 0.9 to 1.5% of the total oxygen uptake proceeds through the formation of the free intermediates of the partial reduction of oxygen.

The concept of the mitochondrial membranes as an important source of intracellular H_2O_2 is supported by the cyanide sensitivity of H_2O_2 steady state levels, a property of the mitochondrial generation of O_2^- and H_2O_2 (24).

The large effect of aminotriazole (Fig. 3) on the H_2O_2 steady state level supports the view of a predominant utilization of H_2O_2 by catalase. Other H_2O_2 consuming enzymes like peroxidase and glutathione peroxidase are also active in the axes early on the imbibition phase. However, they could utilize at most 35% of

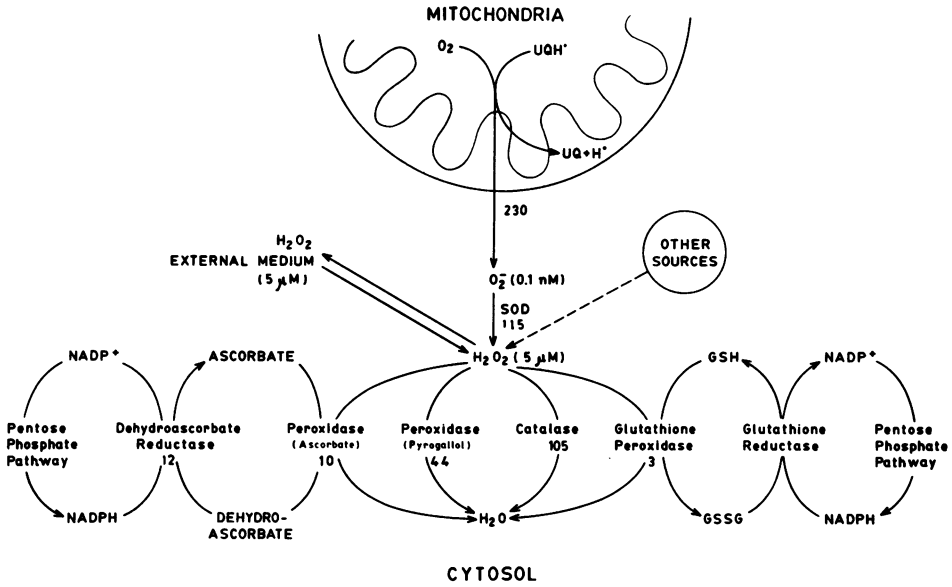

FIG. 4. Scheme of H_2O_2 metabolism in soybean embryonic axes. The numbers indicate the flow of metabolites in pmol/min·axis. UQ, ubiquinone; SOD, superoxide dismutase.

total H_2O_2, assuming saturation with their hydrogen donors (aromatic hydrogen donors, ascorbate, reduced glutathione, etc.). This result agrees with the effect of aminotriazole on the H_2O_2 levels of homogenates from germinating castor beans as reported by Warm and Laties (33).

The estimation of the rate of H_2O_2 utilization by catalase is hindered by insufficient information about the occurrence of extent of the phenomenon of latency (9) and the activity of extramitochondrial sources of H_2O_2 in the soybean axes. The rate of H_2O_2 utilization by catalase appears to be between 102 to 105 pmol/min·axis. The first value corresponds to a situation in which mitochondria are the only source of H_2O_2 and utilization of H_2O_2 by using aromatic hydrogen donors is negligible. The second value is calculated assuming that intracellular H_2O_2 is equal to 5 μM. At any rate, catalase will utilize between 91 and 97% of the intracellular H_2O_2.

The concentration of H_2O_2 measured in the diffusate (about 5 μM) by following both (a) the decrease of an added concentration of H_2O_2 and (b) the increase up to about the same value starting with no H_2O_2 in the medium, seems a reasonable indication of the intracellular H_2O_2 steady state level. The rapid establishment of a steady state situation similar to the intracellular level is in agreement with the large permeability of plant membranes to H_2O_2 (30) and with the rapid effects of aminotriazole and cyanide. The measured H_2O_2 levels are lower than those reported by Warm and Laties (33) in potatoe tubers, tomatoes, and germinating castor beans (224, 92, and 138 μmol/kg, fresh weight, respectively).

The steady state concentration of H_2O_2 is thought to have important physiological implications. A higher H_2O_2 level might affect the axis growth potential by increasing lipid peroxidation and other damaging reactions related to oxyradicals (15). However, the short treatment of the axes with aminotriazole, although substantially increasing the H_2O_2 level did not affect their growth (Table I).

The proposal of Taylorson and Hendricks (32) that the inhibition of catalase by cyanide would lead to an enhanced rate of NADPH oxidation and pentose phosphate activity was based on the implicit assumptions mentioned in the beginning of this paper. So far, no evidence was available to substantiate these assumptions (3). Our results show that H_2O_2 production is an early event and that catalase appears to be the predominant H_2O_2 consuming activity. Moreover, the measured glutathione peroxidase activity offers a cyanide insensitive pathway to oxidize NADPH by H_2O_2. Therefore, an increase in the steady state concentration of H_2O_2 by a treatment with cyanide would only be possible if catalase were more sensitive to the inhibition than the H_2O_2 production system.

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